

# A novel positive tetracycline dependent transactivator (rtTA) variant with reduced background activity and enhanced activation potential

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**Abstract** The tetracycline-controlled transcription system has become one of the most potent systems for experimental manipulations of transcription levels in vivo. Here we report on rtTA variants, which were generated by combining the existing positively regulated Tet repressor domains of rtTA and rtTA-M2 with a modified and multimerized minimal transactivation domain from VP16 (L-domain). A transactivator with multimerized L-domains shows drastically reduced background activity and enhanced transcriptional activation on different tetracycline-responsive promoters. The new rtTA variants require higher doses of doxycycline and display a more linear dose–response curve than the original rtTA or rtTA-M2 proteins. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Doxycycline; VP16; Induction; Tet-On; rtTA

## 1. Introduction

The tetracycline-controlled transcription system (Tet-system [1]) consists of a tetracycline- or doxycycline-dependent transactivator protein and an artificial promoter activated by this transactivator. The transactivator is a fusion protein of the Tet repressor (TetR) of *Escherichia coli* (Tn10) and part of the transcriptional activation domain of Herpes simplex protein 16 (VP16). The target promoter of this transactivator comprises an array of *tetO*-sequences from the Tn10 operon and a minimal core promoter derived from the cytomegalovirus immediate early promoter (CMV). Two types of transactivators have been developed. The tTA (tetracycline-controlled transactivator) binds and activates the operator/responder construct in the absence of tetracycline [1]. Mutants thereof containing amino acid substitutions in the TetR moiety exhibit a reverse phenotype [2,3], i.e. can only activate transcription in the presence of certain tetracycline derivatives. These mutants, called rtTA ('reverse tetracycline-controlled transactivator') and rtTA-M2, require doxycycline or anhydrotetracycline for optimal function. Although both the negatively controlled system and the positively controlled rtTA have been used to study gene function in various organisms [4,5–9], particularly the original rtTA [2] shows some limitations. Among these are a high background activity in the non-induced state due to a residual affinity to *tetO* and a higher instability in some cell

lines and tissues when compared to tTA [3]. Several attempts have been made to improve and modify the Tet system [3,10]. Some derivatives of tTAs exhibited improved properties in activation, doxycycline sensitivity, and stability [3]. By using a multimer of four VP16-derived minimal activation domains [12,13] a new transactivator protein (unchanged in the TetR sequence) was generated whose activation power exceeded that of the original tTA by a factor of 2.3 [10]. This approach has only been described in detail for the tTA system. Since in many biological systems the application of a positively controlled transactivator is desirable (faster onset, easier and cheaper handling for short time induction, especially in transgenic animals), we tried to modify and enhance this kind of transactivator protein. Here we describe such improved rTAs containing multimers of a mutated minimal activation domain. Usage of the earlier described minimal activation domain ('F-domains'), which has been effective for the tTA [10], showed no effect when fused to the rtTA and no improvement was seen for the rtTA-M2. Substantial enhancement was seen using mutated domains lacking the phenylalanine at position 442, even though this residue was previously shown to be essential for activity [12,13].

## 2. Materials and methods

### 2.1. Synthesis of minimal activation domains

The minimal activation domains ('F-domain') were used as described previously [10]. Domains were synthesized by PCR using the 'Tet-On' plasmid (Clontech) as a template. For simplicity we follow the Clontech nomenclature with pUHD17-1neo [2], named Tet-On, indicating the rtTA protein including the complete VP16-domain. Following primers were used: Ffw 5'-TAT ACG CGT TGC ATG CCG ACG CG-3'; Frv 5'-ATG CGC GCC CAA CAT GTC CAG A-3'. The primers introduced an *Mlu*I restriction site at the 5'-end and a *Bss*HII site at the 3'-end, allowing ligation of the resulting fragment into *Bss*HII-digested plasmids pTet-On and prtTA-M2 (for simplicity named M2). *Bss*HII digestion eliminates the VP16-domain in frame. The two enzymes generate compatible cohesive ends. Only with F-domains inserted in the correct orientation was the construct releasable with *Bss*HII at the 3'-end. The stop codon and poly A signal were then reintroduced via a PCR-product with *Mlu*I- and *Pvu*II-ends generated on pTet-On. L-domains arose as a PCR artefact and were multimerized and cloned as described.

### 2.2. Transient transfections

Transfections of NIH/3T3 cells were performed at 60–70% confluence using Rotifect (Carl Roth, Karlsruhe, Germany, 3.0 µl/µg DNA) or Superfect (Qiagen, Hilden, Germany, 5 µl/µg DNA) in 12 well dishes according to the instructions of the distributor. The DNA mixtures contained 0.25 µg of transactivator constructs and in the case of transient/transient transfections 0.25 µg of the Luciferase encoding responder construct pTRE-Luc (Clontech, La Jolla, USA), originally described as pUHC-13-3 [1]. For normalization of trans-

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fection efficiency, 0.015 µg of a pCMV-β-galactosidase plasmid was cotransfected to each well. Doxycycline (Sigma) was added at concentrations of 0–5 µg/ml. Cells were harvested 24 h after induction for luciferase assays.

### 2.3. Luciferase and β-galactosidase assays

Cells were washed once with 2 ml PBS/well and cell lysates were prepared by adding 100 µl of 100 mM potassium phosphate lysis buffer (pH 7.8, 1 mM DTT, 0.2% Triton X-100). 10–50 µl were used to perform luciferase assays as described [14]. Protein content of the lysates was determined according to Bradford [15]. Luciferase activity was measured using an Autolumat LB953 (Berthold, Wildbad, Germany) using the integral mode (20 s) after a delay time of 20 s. β-Galactosidase activity was determined using the 'Galacto-light' kit (Tropix, Bedford, MA, USA) according to the suppliers instructions with measurement performed in the Autolumat LB953 (signal integration over 20 s).

### 2.4. Western blot analysis

NIH/3T3 cells were grown in 92 mm dishes to 70% confluence and transfected transiently with 10 µg of plasmid DNA encoding the different transactivator fusion proteins. After 24 h nuclear extracts of the cells were collected as described in [16]. 50 µg of nuclear protein were separated on SDS-PAGE (10% acrylamide:bis-acrylamide, 29:1) and electroblotted onto Hybond C (Amersham Pharmacia Biotech, Piscataway, NY, USA) blocked in a solution of 10% (v/v) fetal calf serum (Biochrom, Berlin, Germany) in Tris-buffered saline (TBS; 50 mM Tris/150 mM NaCl). For immunochemical detection of the rTA variants a monoclonal 'TetR' antibody (MoBiTec, Göttingen, Germany) was used in TBS containing 2% fetal calf serum.

### 2.5. Electrophoretic mobility shift assay

NIH/3T3 cells were transfected and nuclear proteins were prepared as described in [16]. The *tetO* fragment was generated by annealing of two oligonucleotides resulting in a double-stranded *tetO* fragment with unpaired guanine residues at the 5'-ends of the primers. The protruding ends were labelled with [αP-<sup>32</sup>P]dCTP. Aliquots of the protein extracts (10 of 150 µl) were used as described in [17].

### 2.6. Stable cell lines

NIH/3T3 cells were grown in 92 mm dishes to a confluence of 30–40% and transfected with 10 µg DNA. The transfection assay contained the responder plasmid pTRE-Luc (see Section 2.2) and pTK-Hyg (Clontech) to establish a hygromycin resistance, in a molar ratio of 3:1. Transfection was done as described before. 24 h after transfection the cells were plated to less than 10% confluence. After further 24 h the selection was started by addition of 100 µg hygromycin/ml. Resistant clones were isolated, expanded separately, and analyzed for doxycycline-inducible luciferase activity after transient transfection of the rTA-encoding plasmid pTet-On.

## 3. Results

### 3.1. Fusions between rTetR and minimal activation domains derived from VP16

A minimal activation domain (F) encoding the acidic tran-

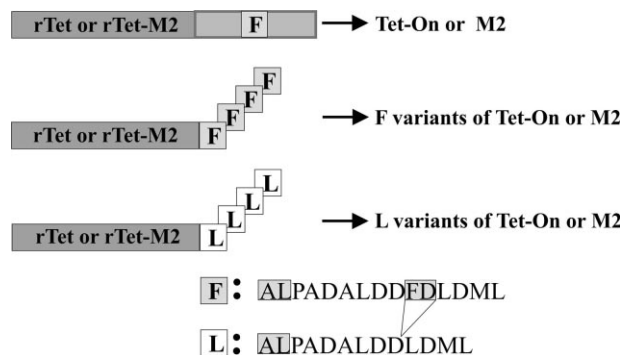


Fig. 1. Replacement of the VP16 activation domain of Tet-On and M2 with minimal activation domains. Scheme of the addition of minimal activation domains to rTetR or rTetR-M2. F denotes the minimal activation domain used by Baron et al. [10], corresponding to the VP16 wild-type sequence positions 436–447. The modified L-domain lacks positions 442 and 443 (phenylalanine and aspartate). Two amino acids at the N-terminus boxed are added due to the cloning strategy.

scriptional activation domain of VP16 (positions 436–447) was synthesized following the approach of Baron et al. [10] to modify the tTA protein. Two to eight copies were fused to the 3'-end of the rTetR or rTetR-M2 domain in frame (Fig. 1). The second type of minimal activation domain (named 'L', for 'lacking') lacked the amino acid positions 442 and 443 (phenylalanine and aspartate) of the original VP16-derived F-domain. One to 20 copies of the L-domain were inserted into pTet-On and pM2 (Fig. 1). Constructs were named according to the number and type (F or L) of minimal activation domain copies. The identity of each construct was confirmed by dideoxysequencing and restriction digestion (Fig. 2A).

### 3.2. Characterization of Tet-On-derived constructs

For characterization of new Tet-On-derived rTA variants we performed Western blot analysis of nuclear extracts of transiently transfected NIH/3T3 fibroblasts (Fig. 2B). In every lane a distinct product can be seen, while the sizes increase according to the number of L-domains. The doxycycline-dependent binding of the rTetR fusion proteins was examined by electrophoretic mobility shift assays. Nuclear protein extracts were incubated with radioactive labelled *tetO* DNA (Fig. 2C). Retarded complexes can be seen in lanes with nuclear protein plus doxycycline. Weaker signals appear without doxycycline with each of the three tested transactivators (Tet-On, 10L, 16L) reflecting residual promoter-binding activity without inducer. The difference between the induced and

Table 1  
Transcriptional activation of TRE-Luc responder by different Tet-On-derived transactivators in double transient transfections

	RLUs (%) – doxycycline	RLUs (%) + doxycycline	Relative activation <sup>a</sup>
Tet-On	10.2(±2.7)	100	10.4(±2.5)
8L	2.4(±0.8)	44.5(±10.4)	19.5(±5.6)
10L	3.0(±2.5)	55.6(±27.8)	24.6(±7.5)
16L	2.4(±2.4)	90.2(±71.5)	40.4(±7.8)
17L	2.8(±1.9)	118.7(±82.5)	43.1(±6.9)
20L	4.4(±3.7)	133.8(±72.1)	34.2(±6.9)
4F	14.2(±6.8)	68.0(±22.6)	4.9(±1.5)
6F	11.4(±2.3)	43.2(±14.7)	3.0(±0.6)
8F	11.6(±4.9)	29.4(±17.0)	2.5(±0.5)

Relative activation factors were measured in double transient transfection assays with the respective transactivators and TRE-Luc with or without doxycycline. Tet-On plus TRE-Luc plus doxycycline was set as 100%.

<sup>a</sup>The average relative activation factor is calculated from values of the single transfection assay and is therefore not calculable from the average RLUs of this table.

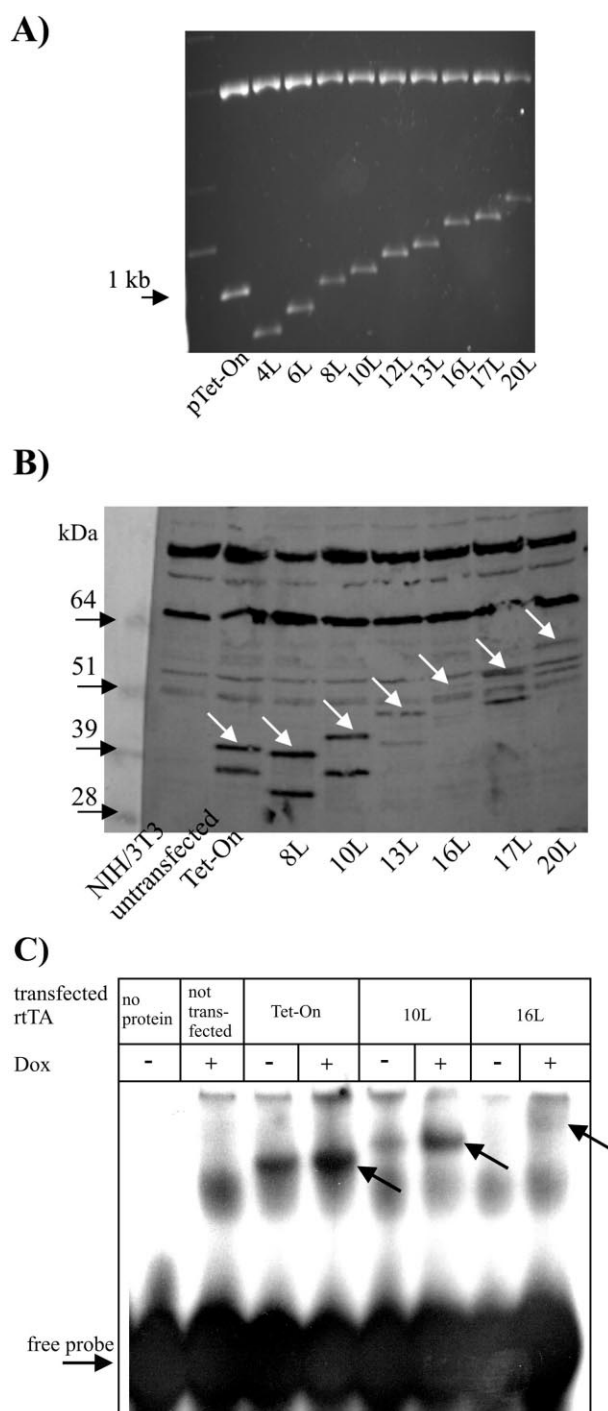


Fig. 2. Characterization of Tet-On (containing the VP16-domain) and Tet-On-derived L-transactivator constructs. A: Separation of *Hind*III-digested DNA of the constructs showing the steps of multimerization with pTet-On on the left site and the corresponding rTetR fusion constructs with increasing numbers of minimal activation domains (L-type). B: Western blot analysis of Tet-On and L-domain constructs. NIH/3T3 cells were transiently transfected with the respective constructs and nuclear extracts were subjected to Western analysis with anti-TetR antibody. C: Characterization of the DNA-binding properties of some rTetR fusions with electrophoretic mobility shift assays. Nuclear cell extracts were prepared from transiently transfected NIH/3T3 cells 24 h after transfection. Extracts (50  $\mu$ g nuclear protein) were incubated with radioactive labelled *tetO* DNA in presence or absence of doxycycline, and complexes were separated electrophoretically.

uninduced state appeared most pronounced in the case of the 16L transactivator.

### 3.3. Activation potential of minimal activation domains

To assess the activation potential of the modified transactivator proteins, NIH/3T3 cells were transfected with the Tet-responsive luciferase reporter plasmid pTRE-Luc together with the respective activator constructs (transient/transient). Luciferase activity was first calculated as relative light units (RLUs)/ $\mu$ g protein and then normalized for transfection efficiency by  $\beta$ -galactosidase activities resulting from uninduced wells. This was necessary because the  $\beta$ -galactosidase activities in the doxycycline-induced wells were reduced to 1/2–1/3 of the uninduced wells. Calculating with reduced  $\beta$ -galactosidase activities would have inadmissibly changed the activation factors. In initial experiments, pools of suspended cells were transfected and split into four (duplicates with and without doxycycline), ensuring identical transfection efficiency for all wells. Since experiments on plated cells showed identical results, we continued to transfect adherent cells because of higher transfection efficiency. Luciferase activity obtained in transient/transient transfections with pTRE-Luc plus pTet-On or pM2 was set as 100%. All other values obtained with the corresponding transactivator variants (uninduced or induced) were related to this activity. Values in Tables 1–3 are mean values from 6–10 transfections.

The F-domain fusions, which were previously reported to be effective in combination with the Tet and the rTet-M2 repressor part [10,3], showed no increased activation potential after addition of doxycycline when compared to the originally described rTetR-VP16 and rTetR-M2-VP16 fusion proteins (pTet-On, prtTA-M2)(Table 1). In addition, the uninduced background of luciferase activity increased upon inclusion of further minimal domains of the F-type. Transactivators with multimers of L-type domains showed no or minimal activation potential when four, five, or six minimal domains were used. A significant improvement was observed with fusions of more than eight L-domains. We observed an increase of the activation potential of Tet-On-derived constructs up to 17 multimerized L-domains, due both to an increase in absolute activity and to a reduced background (Table 1).

Combining the new rTetR-M2 repressor with F-domain multimers reduced activation potentials to about 20% of that seen with the unmodified M2 (not shown). With multimers of L-domains we observed a similar but less strong enhancement as described above for the Tet-On variants (Table 2). However, in contrast to the Tet-On derivatives, the highest increase in activation potential was reached with 11 multimerized L-domains but did not exceed the activation potential of M2 when the reporter TRE-Luc was used. Adding further L-domains led to an increased absolute activity without changing the activation capacity, because background activity also increased.

### 3.4. Characterization of the activation potential of L-domain rtTAs in cell lines containing stably integrated responder constructs

To model a more defined experimental setting, i.e. to provide a defined responder condition and to reduce the luciferase background seen in transient/transient transfections, we used NIH/3T3 cell lines stably transformed with the responder construct pTRE-Luc as host for transient transfection of the

rtTA constructs. Different stable clones were transfected with plasmids encoding the original transactivator (Tet-On, i.e. rTetR-VP16) or with DNA encoding some of the L-domain variants. Three TRE-Luc-containing clones, numbers 21, 29, and 30, were chosen to show differences in induction properties of luciferase activity caused by differences in copy number and chromosomal integration site. In every case the activation factor of luciferase activity was higher in these transient/stable transfection assays than in transient/transient transfections (Tables 1 and 3). L-domain transactivators gave higher activation factors than did Tet-On (Table 3). The increase in activation was specific for each of the responder clones. The best performing constructs (rTetR-16L, rTetR-17L) gave a ca. 10-fold larger activation factor compared to Tet-On in clones 21 and 30, and ca. 2.5-fold higher in clone 29. This increase in activation factor was caused in part by higher induced activity in clones 21 and 30 (2.8–4.3-fold more than given by Tet-On), and by lower uninduced background activity in all three clones.

### 3.5. rtTA-dependent transactivation from plant-derived *tetO* promoter constructs

To further explore the capability of our new transactivators, we used two newly developed *tetO*-containing promoter constructs (Tax-Luc and TopFree-Luc) based on plant virus CaMV minimal promoter sequences [18,19](Table 2). As in the previous experiments, activity of the original Tet-On or M2 (RLU/ $\mu$ g protein normalized by  $\beta$ -galactosidase activity) with the respective responder was set as 100% and all other activities were related to this value. Background activity (which combines basal activity of the promoter alone plus residual activity of the transactivator without doxycycline) is lower for the plant-derived promoters than for the TRE-Luc responder with any of the transactivators, and is almost non-existent with the L-domain transactivators. This results in very high relative activation factors of 300–600 (in transient/transient transfections). However, the absolute activity which can be achieved in NIH/3T3 cells transiently cotransfected with Tet-On-17L and either Tax-Luc or TopFree-Luc is only about 70–90% of than seen with Tet-On activity. In

case of the modified M2 we reach 50–60% of M2 activity with the M2-11L. In general the L-variants gave much higher activation factors than did the VP16-containing transactivators (Tet-On and M2) when they were combined with the plant-derived *tetO* promoter constructs.

### 3.6. Response of different transactivators to varying concentrations of doxycycline

To compare the response of the original transactivators and the L-variants to varying doxycycline concentrations, we transfected the best inducible responder-containing clone (number 30, Table 3) with the respective activator construct and measured luciferase activity after incubation with different concentrations of doxycycline (Fig. 3). Activation of the original Tet-On occurred in a very narrow concentration window, with maximum induction reached at 0.5  $\mu$ g doxycycline/ml. The dose–response curve for the 17L was much less steep, and almost linear from 0.1 to 1  $\mu$ g doxycycline/ml, with ca. 2  $\mu$ g/ml being required for full activation. Full activation of the M2 and M2-11L was obtained using approximately 0.2  $\mu$ g/ml and 1  $\mu$ g/ml doxycycline, respectively, with the curve for the L-derivative again being less steep.

## 4. Discussion

The primary goal of this study was to improve the activation potential of the positive tetracycline (doxycycline) -dependent transactivator (rtTA) [2]. Similar to the approach described by Baron et al. [10] for modification of the tTA we fused the same minimal activation domain derived from VP16 to the rtTA and the rtTA-M2 DNA-binding domain. Gene synthesis PCR reactions produced both the actual F-domains and a mutant thereof (presumably as a PCR artefact), which we called the L-domain. This mutant lacked two amino acids (positions 442 and 443), including Phe<sup>442</sup>, which had been reported to be of crucial importance for function of the VP16 transcription activation domain [12,13]. Multimers of the minimal activation domains (F-domains), which were described to be functional when fused to the TetR [10], showed no effect when fused to reversed TetR. In fact, the background

Table 2  
Transcription activation in NIH/3T3 cells transiently transfected with different activator and responder constructs

Transactivator	RLUs (%) – doxycycline	RLUs (%) + doxycycline	Activation factor <sup>a</sup>
TRE-Luc			
Tet-On	10.2(±2.7)	100	10.4(±2.5)
16L	2.4(±2.4)	90.2(±71.5)	40.4(±7.8)
17L	2.8(±1.9)	118.7(±82.5)	43.1(±6.9)
M2	1.2(±0.5)	100	89.8(±38.6)
M2-11L	0.6(±0.04)	42.8(±13.4)	72.1(±23.9)
M2-16L	1.0(±0.5)	58.9(±15.9)	70.3(±11.5)
Tax-Luc			
Tet-On	3.01(±2.1)	100	56.6(±31.3)
16L	0.17(±0.1)	84.8(±29.9)	401.9(±156.6)
17L	0.26(±0.1)	100.4(±63.1)	508.0(±385.3)
M2	0.4(±0.1)	100	221.8(±43.5)
M2-11L	0.06(±0.02)	36.3(±10.8)	541.3(±109.2)
TopFree-Luc			
Tet-On	2.66(±1.57)	100	54.6(±18.5)
16L	0.33(±0.15)	69.5(±7.1)	333.2(±72.6)
17L	0.27(±0.15)	67.8(±25.7)	338.2(±154.3)
M2	0.5(±0.2)	100	191.8(±59.8)
M2-11L	0.08(±0.02)	53.0(±10.8)	642.8(±198.3)
M2-16L	0.1(±0.04)	82.3(±20.6)	553.6(±166.4)

<sup>a</sup>The average relative activation factor is calculated from values of the single transfection assay and is therefore not calculable from the average RLUs of this table.

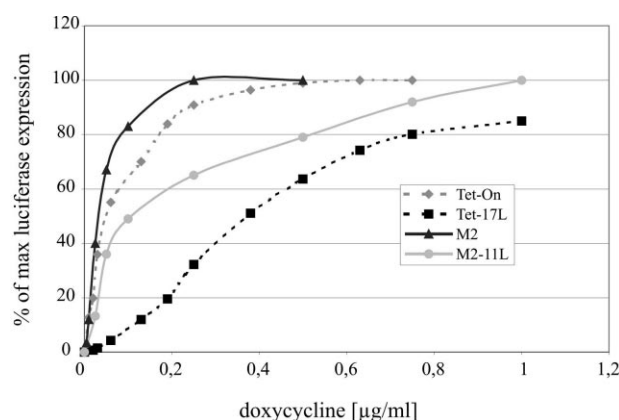


Fig. 3. Doxycycline requirement of Tet-On, 17L, M2 and M2-11L. The stable responder clone TRE-Luc 30 (see Table 3) was transiently transfected with a single transactivator construct and was cultivated with different concentrations of doxycycline. Luciferase activity was measured 24 h after transfection. 5 µg doxycycline/ml medium was set as 100% activation.

activity (non-induced state) increased with increasing numbers of F-domains. Urlinger et al. reported a similar observation [3]. In contrast to this, multimers of the mutant minimal activation domain (L-domains) displayed a steady increase of doxycycline-dependent transcription activation (Table 1). This is mainly due to a strongly reduced background expression from the *tetO7* promoter in the uninduced state. With more L-domains we observed a slight increase of background activity, but the absolute response upon induction was still higher than with Tet-On, resulting in an optimum of transcriptional activation at 16–17 L-domains (Table 1). Tet-On/L-domain constructs exhibited superior activation properties both in transient/transient and in transient/stable assays with different Tet-responsive promoters (Tables 2 and 3).

When we fused L-domains with rTetR-M2 [3], an improved reversed TetR version, we found an optimal of transcriptional activation with 10–12 L-domains and an increase in background with higher numbers of L-domains. At least with TRE-Luc as responder this activation is slightly below that of the VP16-containing M2. We did not see any significant activation potential with M2/F-domain constructs. Urlinger et al. [3] described a 3F-domain to be an equally effective transactivator domain as VP16. However, this was in the context of rTetR<sup>S</sup>-M2, a codon usage optimized version. Our experi-

ments were performed with the non-humanized M2 version to allow a comparison with the original rTA version.

β-Galactosidase activities from induced cells (plus doxycycline) were reduced to about 50% of the activity from uninduced samples. This may be a squelching effect arising from depletion of general transcription factors through non-specific formation of transcription complexes by the VP16-domain (independently from tet-promoter sequences and doxycycline). Squelching has been described for GAL4 [11] and VP16 domains, and has been suggested for tTA because this transactivator is only tolerated in limited amounts by cells [4]. Since the rTA protein has been described as inherently unstable [3] and β-galactosidase repression occurs when doxycycline is added, we speculate that doxycycline might stabilize this transactivator and thus potentiate the squelching effect.

Electrophoretic mobility shift assays with Tet-On/L-domain constructs (Fig. 2C) demonstrated a reduced background affinity (without doxycycline) of L-domains for the *tetO7* sequence element. Note that in Fig. 2B,C similar amounts of cellular protein and (according to the Western blot) comparable amounts of reverse transactivator resulted in clearly reduced signals in the band shift experiment with 10L. According to our results Phe<sup>442</sup> seems not to be essential for transactivation activity of the VP16 minimal domain. Since even the absolute reporter activity with 8F is lower than with 8L (Table 1) it seems that in our system the loss of the Phe<sup>442</sup> residue in fact is beneficial for the activation power of the whole complex.

Highest induction rates could be achieved by using novel Tet-responsive promoters designed by Böhner et al. [18,19]. As shown in Table 3, the 16L transactivator again is superior to the original Tet-On, giving activation with almost no background even in transient transfection. However, absolute strength of reporter activity is diminished in this combination. Thus, in our hands highest activation factors with the variants of Tet-On were obtained with the combination of 16L/Tax-Luc and highest absolute RLU values were obtained with 20L/TRE-Luc.

In case of the rTetR-M2, an L-domain-based improvement of the activation potential was only observed when the compared transactivators M2 (rTetR-M2-VP16) or M2-11L (rTetR-M2-11L) were cotransfected with the responder constructs Tax-Luc or TopFree-Luc. This improvement was weaker than that seen for the rTetR variants (i.e. Tet-On versus 17L). However, this result fits well with the observation that L-domains were able to improve the activation potential

Table 3

Transcription activation in stably transformed NIH/3T3 responder cell lines by transient transfection of different Tet-On derived activator constructs

Transactivator	Responder (TRE-Luc, stable)	RLUs (%) – doxycycline	RLUs (%) + doxycycline	Activation factor <sup>a</sup>
Tet-On	clone 21	2.1(±0.8)	100	54.8(±19.6)
16L	clone 21	0.5(±0.4)	282.2(±77.3)	639.2(±287.6)
Tet-On	clone 29	2.1(±0.8)	100	42.4(±12.6)
16L	clone 29	0.8(±0.3)	80.0(±24.1)	106.0(±54.5)
Tet-On	clone 30	3.2(±0.9)	100	33.3(±7.5)
10L	clone 30	1.0(±0.5)	118.0(±22.1)	148.3(±94.0)
16L	clone 30	1.6(±0.8)	430.7(±215.4)	287.3(±63.6)
17L	clone 30	1.6(±1.0)	380.6(±151.7)	256.2(±60.3)

Activation factors were measured by transient transfection of indicated transactivators in different stable responder clones. Tet-On plus TRE-Luc plus doxycycline was set as 100% and is specific for each responder line.

<sup>a</sup>The average relative activation factor is calculated from values of the single transfection assay and is therefore not calculable from the average RLUs of this table.

of the rTetR-based constructs in combination with the responder TRE-Luc and not, when combined with rTetR-M2.

The dose–response curve for doxycycline showed that the L-domain derivatives require more doxycycline for maximal activation than do the Tet-On or M2 constructs, and that the activation curves span a considerably wider range of concentrations (Fig. 3). This feature might be of value if truly dose-dependent and reproducible inductions are needed. It might, on the other hand, be problematic to reach such high doxycycline levels in transgenic animals. A levelling off of the dose–response curve can also be detected comparing the M2 with the corresponding L-domain variant (M2-11L). As the M2 protein is more sensitive to doxycycline than Tet-On, M2-11L also shows an earlier maximum activity than Tet-17L.

The observation that a modification of the activation domain affects the ligand-binding properties of the transactivator seems surprising and cannot be explained by the data available. It may be speculated that the L-multimer interacts with the doxycycline-binding domain, possibly leading to a mutual masking of doxycycline-binding site and DNA-binding domain. A reduced DNA-binding activity and hence reduced background would result if no doxycycline was present. Addition of relatively large amounts of doxycycline would saturate the doxycycline-binding domain, and thus liberate the DNA-binding domain.

The evident reduction of background activity seen with the L-domain activators could add to the existing tools of Tet-dependent inducible transactivation systems. The benefits of the L-domains are most obvious in combination with rtTA constructs. M2/L domains perform better than M2 alone combined with the plant virus-derived responders. This is of importance because the advantage of strongly reduced basal activities is supported with L-domain-containing transactivators. This is an advantage that contributes to experiments dealing, for example, with the expression of suicide genes, as described by Leuchtenberger et al. [20].

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